Supporting Information

Photostable and Proteolysis-Resistant Förster Resonance Energy Transfer-Based Calcium Biosensor

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Experimental Procedure:

Expression and Purification of SNAP-Twitch-2B-CLIP (STC):
Plasmids encoding STC were transformed into chemically competent BL21(DE3)* *E. coli* cells. After the cells were transferred to LB plates supplemented with carbenicillin (0.050 mg/mL), the cells were incubated at 37 °C for 12-18 h. An individual colony from the transformation plate was inoculated with LB (70 mL) supplemented with carbenicillin (0.050 mg/mL). The flasks were incubated at 37 °C with shaking at 220 rpm for 12-18 h. This seed culture (15 mL) was transferred into LB (1 L) supplemented with carbenicillin (0.050 mg/mL) before incubation at 37 °C with shaking at 220 rpm. Upon reaching a cell density of OD_{600} between 0.50 and 0.56, the culture was induced through addition of IPTG (0.5 mM) before incubation at 25 °C with shaking at 225 rpm for 6 h. The cultures were centrifuged at 6084.1 rcf at 4 °C for 20 min. The supernatant was discarded, and the cell pellet was resuspended in lysis buffer (20 mL of 50 mM HEPES buffer, 300 mM NaCl, 10 mM β-mercaptoethanol (βME), pH 7.6). A protease inhibitor cocktail (HALT) was added to the suspended cells at the manufacturer's recommended concentration, and the cells were lysed by sonication at a 50% amplitude pulsation (1 s on and 1 s off) for a 1-min cycle and a 2-min rest between each cycle, for a total of 5 cycles. The lysate was centrifuged at 26891.1 rcf at 4 °C for 45 min. The supernatant was loaded onto a nickel-charged microporous polymethacrylate resin (MIDA, Purolite). The supernatant underwent batch binding with the charged resin overnight in 4 °C with a slow tilt rotation.
After batch binding, the mixture was homogenized by inversion and applied to a gravity column. Flow through, wash, and elution fractions were collected using the lysis buffer, lysis buffer with imidazole (40 mM), and lysis buffer with a higher concentration of imidazole (250 mM), respectively. The fractions containing the desired protein was further purified using fast protein liquid chromatography (FPLC) using a Superdex^{TM} 75 10/300 GL column (GE Healthcare). The column was washed with 1 volume of 20% ethanol and 1 volume of double-distilled water and pre-equilibrated with 1 column volume of a size exclusion reference buffer (RB, 50 mM HEPES, 50 mM NaCl, 10% glycerol, 10 mM βME, pH 7.6) at a flow rate of 0.2 mL/min. The protein was eluted with the RB at 0.2 mL/min. A 12% polyacrylamide SDS-PAGE gel confirmed the homogeneity of the protein.

Bioconjugation of Fluorophores onto STC:
At 37 °C for 1 h in the dark, the purified protein was incubated with BG-Alexa Fluor 488 and BC-Alexa Fluor 546 dyes (New England Biolabs) in a 1:2:2 molar ratio (protein:BG-dye:BC-dye) in DMSO with the addition of dithiothreitol (DTT, 1 mM). Unbound dye was removed by microfiltration of the solution with a 10 kDa molecular weight cut off (MWCO) microfilter (Amicon Ultra-0.5 Centrifugal Filter Unit) at 17136 rcf at 4 °C for 5 min.

Expression and Purification of Twitch-2B:
Using BL21(DE3) (Thermo Scientific^{TM}) chemically competent *E. coli* cells, the miniprepped CFP-Twitch2B-YFP plasmids were heat shock transformed. The cells were plated on warm LB agar plates supplemented with carbenicillin (50 mg/mL) and placed in a 37 °C incubator to grow for 12-18 h. Seed cultures were made by picking single colonies from the transformation plates and inoculating into a culture tube with 5 mL LB and 5 µL of carbenicillin (50 mg/mL). The tubes were left to grow at 37 °C, shaking at 220 rpm, for 6 h. An expression culture was made by adding the seed culture in 1 L of LB and 1 mL of carbenicillin (50 mg/mL). The expression culture was left to grow at 37 °C, shaking at 220 rpm, until the OD_{600} is at 0.6. Once it has reached this OD_{600}, the culture was induced with isopropyl-β-D-thio-galactoside (IPTG, 1 mM). The expression culture was moved to a pre-cooled 25 °C incubator for induction at 225 rpm for 12 to18 h.
The culture was removed from the incubator and centrifuged at 6084.1 rcf at 4 °C for 20 min to harvest the cells. The cells were resuspended in 20 mL of lysis buffer (25 mM MOPS buffer, 100 mM KCl, pH 7.6). A protease inhibitor cocktail (HALT) was added to the suspended cells at the manufacturer's recommended concentration. The suspended cells were lysed by sonication at a 50 % amplitude pulsation (1 second on and 1 second off) for a 1-min cycle and a 2-min rest between each cycle, with a total of 3 cycles. The lysate was centrifuged at 26891.1 rcf at 4 °C for 45 min. The supernatant was loaded onto a nickel charged immobilized metal affinity chromatography (IMAC) resin (1.5 mL of IMAC charged with $NiSO_4$ and equilibrated with the lysis buffer) The supernatant underwent batch binding to the charged IMAC resin overnight in 4 °C with constant shaking.
After batch binding, the IMAC/supernatant mixture was homogenized by inversion and loaded into a gravity column. The following fractions were collected: a flow through fraction (FT), a wash fraction using a solution of lysis buffer with 25 mM imidazole (W), and elution fractions using a solution of lysis buffer with 250 mM imidazole (E1-5). SDS-PAGE confirmed the expression of the protein and its purity. Ethylenediaminetetraacetic acid (EDTA, 0.5 M) was added to the eluted fractions containing the protein of interest and the mixture was dialyzed in 4 L of lysis buffer overnight and concentrated to approximately 1 mL.

Tagging Insulin with N-Hydroxysuccinimide Ester (NHS)-Alexa Fluor 488:
In order to label insulin with Alexa Fluor 488, Alexa Fluor 488-NHS ester (Thermo Fisher Scientific) and recombinant human insulin were combined at final concentrations of 20 µM and 84 µM, respectively, in a total volume of approximately 180 µL. The volume was split into 30 small reactions and incubated in a thermocycler for 1 h at 4 °C. The total volume of the reaction was serially dialyzed for 3 h and then overnight in 2 L of PBS pH 8.0, such that unbound, hydrolyzed NHS-Alexa Fluor 488 could be removed from the labelled insulin solution.

Fluorescence Imaging of SDS-PAGE Gels:
Tagging of SNAP- and CLIP-tags with Alexa Fluor 488 and Alexa Fluor 546 was visualized by imaging the fluorescently tagged proteins on a 0.75 mm thickness SDS-PAGE using a Typhoon Trio+ Scanner (GE Healthcare) in "fluorescence acquisition" mode Fluorescent signals from Alexa Fluor 488 was imaged using the Green (532 nm) laser and the 526 SP filter at 500 PMT voltage, whereas for detecting Alexa Fluor 546, the Green (532 nm) laser and 580 BP filter at 400 PMT voltage was used. Images were recorded at 25-micron pixel size for high resolution scanning. Once fluorescent signals were imaged, the total protein was visualized using Coomassie dye.

Fluorescent Intensity Measurements by TECAN Spark Plate Reader:
In a Corning black polystyrene 96 well plate, 100 µL of buffer (either TB or RB) was used to fill the wells unless otherwise stated. 4 µL of protein sensor solution was added to each well, and 405 nm and 450 nm excitation light were used excite the donor fluorophores on Twitch-2B and STC, respectively. Emission spectra from 450 to 600 nm and 500 to 600 nm were collected for Twitch-2B and STC. Excitation and emission bandwidths were set to 5 nm.

Determining Protein Concentration using the Bradford Assay:
Protein concentration was determined with the Bradford protein assay. BSA standards and the protein samples were introduced to Coomassie Brilliant Blue G-250 dye (Bio-Rad) for protein binding in 96 well Costar plate (SKU#3595), which undergoes a colorimetric change. Absorbance is measured at 595 nm, and the protein concentration is determined through interpolation of the standard curve.

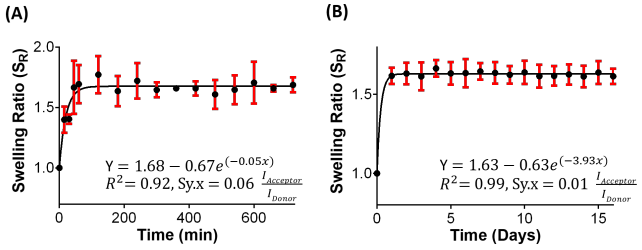

Figure S1. Swelling studies of PEGDMA 2000.
PEGDMA 2000 hydrogels were allowed to swell in PBS, pH 7.4 at room temperature to reach a steady state mass. To quantify the degree of swelling, the swelling ratio of the hydrogel was calculated. The formula is shown in equation 1

$$S_R = \frac{W_s}{W_i} * 100 \qquad \text{Equation 1}$$

where S_R is the hydrogel swelling ratio, W_s is the hydrogel swell mass after incubation in PBS, pH 7.4 at room temperature and W_i is the initial hydrogel mass right after polymerization.[1] The results indicate that (A) the hydrogel reaches a steady state mass after 5 h of incubation after which (B) there is no change in S_R for up to two weeks (relative standard deviation (RSD) = 0.87%).

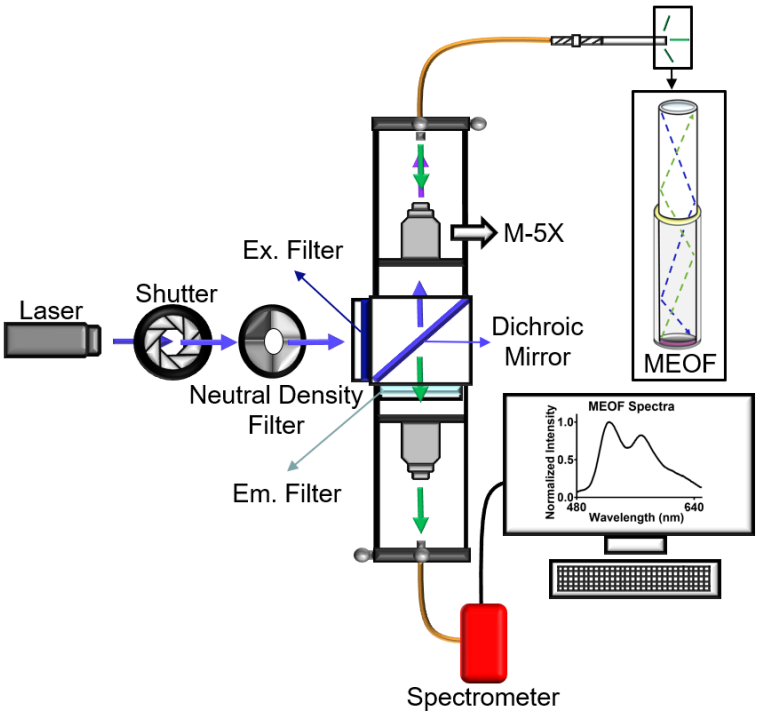

Figure S2. Spectroscopic Benchtop System. A benchtop optical system was constructed for MEOF excitation and emission collection. The system was integrated into a cage system to maintain fiber alignment. In the case of STC, excitation light is provided by a 450 nm continuous wave laser (4.5 mW, CPS450 ThorLabs) that is mechanically shuttered (CX2450B, NM Laser Product). The beam passes through a variable neutral density filter wheel (NDC-25C-4, Thorlabs) to reduce power in the beam. The beam then passes through a filter cube block (CM1-DCH with attached SM30 circular filter mounts tubes, Thorlabs), comprising a 450 nm bandpass filter (ET448/19x, Chroma) tuned to the laser wavelength, a dichroic beam splitting mirror with center wavelength at 458 nm (ZT458rdc, Chroma), and a 465 nm long pass filter (AT465lp, Chroma) in the emission pass to filter out any back-reflected laser light. Laser light reflected off the dichroic mirror is focused by a M-5x microscope objective lens (0.10 NA, Newport) into a SMA-SMA optical fiber patch cable (M92L01, Thorlabs). This optical fiber is attached to a SMA female plate mounted within a XY translation stage (ST1XY-S Translation Mount, Thorlabs) for fine X-Y alignment of the optical fiber relative to the focused laser beam. The MEOF is coupled to the distal end of the patch cable by a SMA to SMA mating sleeve (ADASMA, Thorlabs) Fluorescence emission is collected by the MEOF and collimated into our cage system, passing through the dichroic mirror and the 465 nm longpass filter, where it is coupled into an optical fiber. This coupling uses the same strategy as for the laser light into the MEOF system. The distal end of the optical fiber directs light into a spectrometer (CCS200, Thorlabs). ThorLabs software collects spectrometer data that is subsequently analyzed in MATLAB (MathWorks). In the case of the Twitch-2B construct, the laser, excitation, dichroic, and emission filters were replaced with a 405 nm continuous wave laser (Newport LQA305-40P laser, 45 mW maximum power), FBH405-10, DMLP425R, and FELH0450 filters (Thorlabs) respectively.

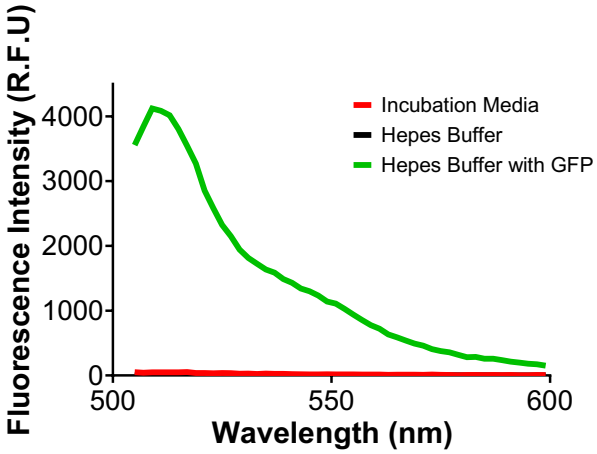

Figure S3. GFP encapsulation within the MEOF. GFP was loaded into the sensor-matrix of the MEOF probe and incubated in HEPES buffer for 5 h. 150 µL of the HEPES buffer was then analyzed with a fluorescence plate reader (red). For the experiment, negative and positive controls include 150 µL of HEPES buffer without GFP (black) and 150 µL of HEPES buffer spiked with 0.1 µL of GFP at 4 mg/mL (green). The spectral curves demonstrate GFP encapsulation within the MEOF.

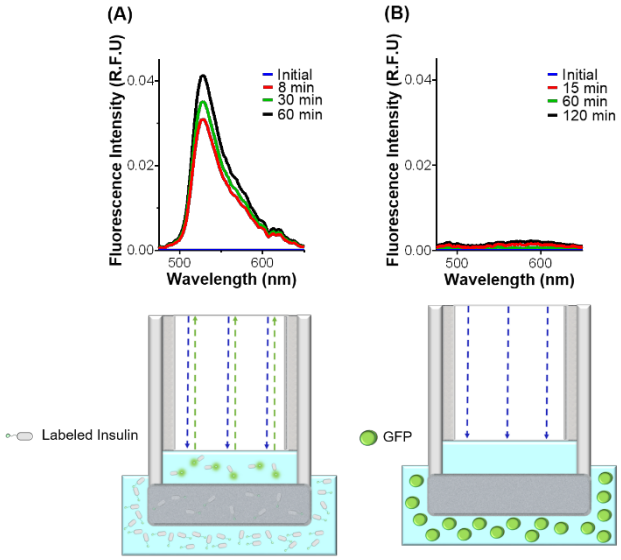

Figure S4. Selective permeability of the filter-membrane. The spectral curves demonstrate GFP encapsulation within the MEOF. Diffusion spectra (top) and schematic representation (bottom) of fluorescently labeled (A) insulin and (B) GFP demonstrate selective permeability by the MEOF.

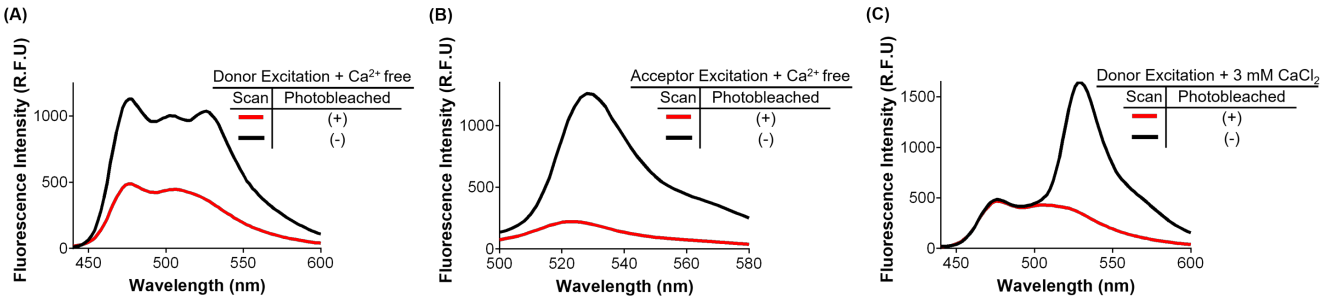

Figure S5. FRET properties of non-photobleached and photobleached Twitch2B samples. Both solutions contained 150 µL of Twitch2B protein (4 mg/mL). For the photobleached samples, both (A) donor excitation at 405 nm and (B) direct acceptor excitation at 475 nm have an absent acceptor fluorescence signal. (C) Moreover, donor excitation with the introduction of Ca^{2+} does not increase the FRET efficiency in the photobleached sample.

ATGCGGGGTTCTCATCATCATCATCATCATGGTATGGACAAAGACTGCGAAATGAAGCGCACCACCCTGGAT
AGCCCTCTGGGCAAGCTGGAACTGTCTGGGTGCGAACAGGGCCTGCACCGTATCATCTTCCTGGGCAAAGG
AACATCTGCCGCCGACGCCGTGGAAGTGCCTGCCCCAGCCGCCGTGCTGGGCGGACCAGAGCCACTGATGC
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ACCACCCAGTGTTCCAGCAGGAGAGCTTTACCCGCCAGGTGCTGTGGAAACTGCTGAAAGTGGTGAAGTTC
GGAGAGGTCATCAGCTACAGCCACCTGGCCGCCCTGGCCGGCAATCCCGCCGCCACCGCCGCCGTGAAAAC
CGCCCTGAGCGGAAATCCCGTGCCCATTCTGATCCCCTGCCACCGGGTGGTGCAGGGCGACCTGGACGTGG
GGGGCTACGAGGGCGGGCTCGCCGTGAAAGAGTGGCTGCTGGCCCACGAGGGCCACAGACTGGGCAAGCC
TGGGCTGGGTCGCATGCAAGTTGCCGACGCCAGCGAAGAGGAACTGAGCGAGTGCTTCAGAATCTTCGACT
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GACGAGGACGTGGACGAGATCTTCGGCGACTCCGACACCGACAAGAACGGCAGAATCGATTTCGACGAGTT
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GCAAAGGAACATCTGCCGCCGACGCCGTGGAAGTGCCTGCCCCAGCCGCCGTGCTGGGCGGACCAGAGCCA
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GCCCTGCACCACCCAGTGTTCCAGCAGGAGAGCTTTACCCGCCAGGTGCTGTGGAAACTGCTGAAAGTGGT
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TGAACACCGCCCTGGACGGAAATCCCGTGCCCATTCTGATCCCCTGCCACCGGGTGGTGCAGGGCGACAGC
GACGTGGGGCCCTACCTGGGCGGGCTCGCCGTGAAAGAGTGGCTGCTGGCCCACGAGGGCCACAGACTGGG
CAAGCCTGGGCTGGGT

Legend: HisTag Snap Tag Linkers Twitch-2B CLIP tag

Figure S6. Nucleotide sequence encoding the STC protein.

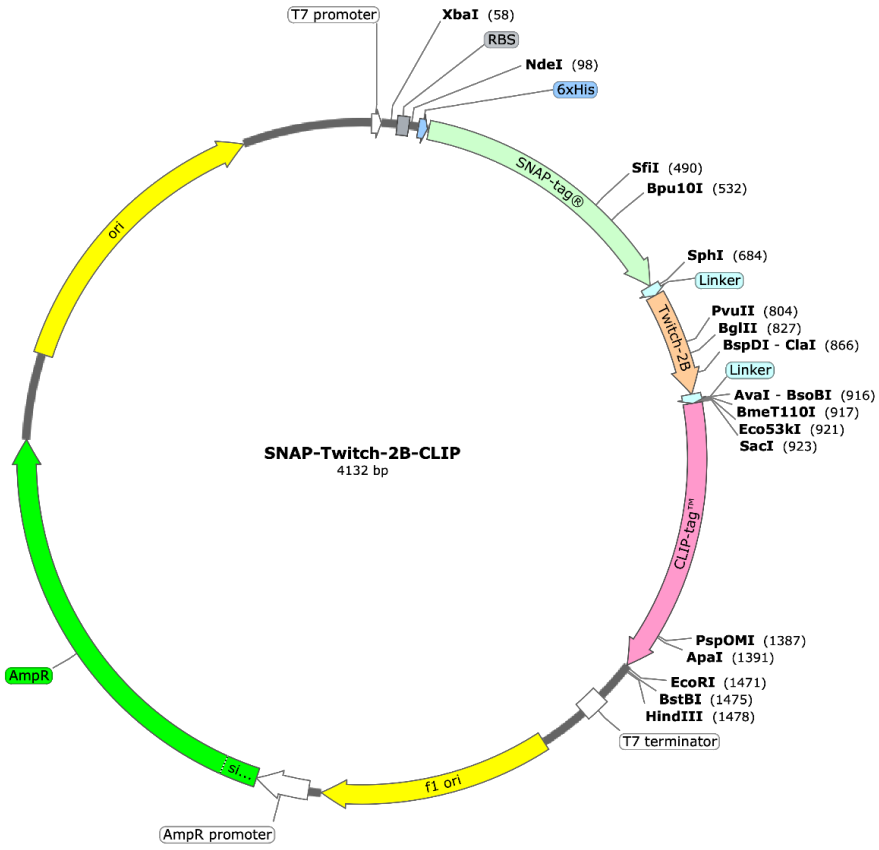

Figure S7. Plasmid Map of the gene encoding the STC protein in the pRSETB vector.

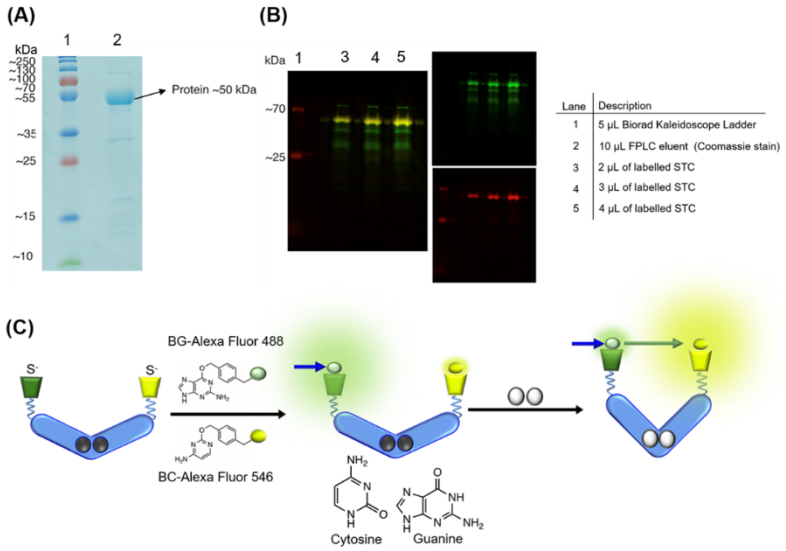

Figure S8. Production of STC. (A) SDS-PAGE was used to analyze the FPLC fractions to show the high protein yield and purity of STC. After STC protein was fluorescently labelled with Alexa Fluor 488 and 546 dyes using orthogonal SNAP and CLIP tags, which was confirmed with a (B) Typhoon fluorescence scanner.[2-4] The covalent linkage of the Alexa Fluor dyes onto SNAP and CLIP tag are shown in (C) where cytosine and guanine are displaced in the reaction.

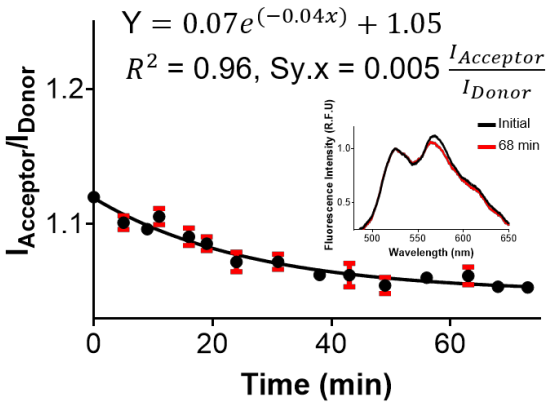

Figure S9. Unbinding of Ca^{2+}. The $I_{acceptor}/I_{donor}$ ratio falls after media exchange from RB with $CaCl_2$ (1.25 mM) solution to calcium-free RB. *Inset:* Emission spectra at times 0 and 68 min.

References:

(1) Leach, S. P. Z. and J. B. Hydrolytically Degradable Poly(Ethylene Glycol) Hydrogel Biomacromolecules. *Biomacromolecules* 2011, *11* (5), 1348–1357. https://doi.org/10.1021/bm100137q.Hydrolytically.

(2) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. A General Method for the Covalent Labeling of Fusion Proteins with Small Molecules in Vivo. *Nat. Biotechnol.* 2003, *21* (1), 86–89. https://doi.org/10.1038/nbt765.

(3) Keppler, A.; Kindermann, M.; Gendreizig, S.; Pick, H.; Vogel, H.; Johnsson, K. Labeling of Fusion Proteins of O6-Alkylguanine-DNA Alkyltransferase with Small Molecules in Vivo and in Vitro. *Methods* 2004, *32* (4), 437–444. https://doi.org/10.1016/j.ymeth.2003.10.007.

(4) Keppler, A.; Pick, H.; Arrivoli, C.; Vogel, H.; Johnsson, K. Labeling of Fusion Proteins with Synthetic Fluorophores in Live Cells. *Proc. Natl. Acad. Sci. U. S. A.* 2004, *101* (27), 9955–9959. https://doi.org/10.1073/pnas.0401923101.